Imbalance between nitric oxide generation and oxidative stress in patients with peripheral arterial disease: Effect of an antioxidant treatment

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Background: **Nitric oxide (NO), a potent vasodilator produced by endothelial cells, is reduced in patients with peripheral arterial disease (PAD), but the mechanism has not been fully elucidated. Because NO is rapidly inactivated by superoxide anion, we speculated that enhanced oxidative stress could lower NO generation. The aim of our study was to investigate if an imbalance between oxidative stress and NO does exist in patients with PAD and if an increase of NO formation could be achieved by an antioxidant treatment.**

Methods: **In a first study, serum levels of nitrite and nitrate (NOx), markers of NO generation, and 8-hydroxy-2-deoxyguanosine (8-OHdG), a marker of oxidative stress and maximal walking distance (MWD), were measured in 40 PAD patients and 40 controls. In a second study, 10 PAD patients were randomly allocated in a crossover design to intravenous propionyl-L-carnitine (6 g/day) or placebo for 7 days, with a washout of 30 days between the two phases of the trial. Serum levels of NOx and 8-OHdG were measured before and after the study.**

Results: **Compared with controls, serum levels of 8-OHdG (mean ± SD) were significantly increased in PAD patients (4.4 ± 3.1 ng/mL vs 2.4 ± 1.2 ng/mL; $P < .001$), and serum levels of NOx were significantly decreased (11.6 ± 6 μM vs 17 ± 6.1 μM; $P < .001$). Levels of 8-OHdG and NOx were inversely correlated ($r = -0.879$; $P < .001$). Serum levels 8-OHdG were inversely correlated with MWD ($r = -0.48$, $P = .002$). The interventional trial showed no changes in the patients given placebo. Patients treated with propionyl-L-carnitine showed a significant increase of MWD from 101 ± 31 meters to 129 ± 35 meters ($P = .007$) and in NOx from 14.5 ± 4.5 μM to 17.1 ± 3.8 μM ($P = .007$). A significant decrease of 8-OHdG from 3.6 ± 1.1 ng/mL to 2.6 ± 1 ng/mL was also found ($P = .005$.)**

Conclusions: **This study suggests that in PAD patients, the reduction of NO generation could be dependent upon enhanced oxidative stress. (J Vasc Surg 2006;44:525-30.)**

Peripheral arterial disease (PAD) is caused by atherosclerosis of the leg arteries and is usually complicated by vascular accidents occurring not only in peripheral circulation but also in the cerebral and coronary trees.[1] Approximately one third of patients with PAD have claudication, that usually deteriorates slowly. Claudication worsens in 25% of patients and about 5% undergo amputation ≤5 years.[2]

Nitric oxide (NO) is synthesized from L-arginine and is constitutively released by endothelial cells whereby it serves to regulate vascular tone and inhibit platelet function.[3] NO is a potent antiatherosclerotic molecule, as indicated by experimental study showing that L-arginine supplementation reduces atherosclerotic progression.[4] NO generation is reduced in patients with PAD, suggesting that it may be implicated in the atherosclerotic progression,[5] but the mechanisms accounting for its lowered generation have not been fully elucidated. In particular, it has not been clarified if the reduced generation of NO could be dependent on enhanced oxidative stress that may reduce NO generation via its accelerated degradation or inhibition of NO synthase.[6]

Enhanced serum levels of isoprostanes and autoantibodies against oxidized low-density-lipoprotein have been detected in patients with PAD,[7,8] suggesting that PAD patients have high oxidative stress. However, to our knowledge, whether a relationship between NO generation and oxidative stress exists has never been explored in this clinical setting. Therefore, the first aim of the study was to investigate whether an imbalance between oxidative stress and NO does exist in patients with PAD. The second aim of the study was to analyze whether an increase of NO formation could be achieved by an antioxidant treatment. To this purpose an interventional trial was done with propionyl-L-carnitine (PLC), a molecule that has been shown to reduce cellular superoxide anion (O_2^-) production through inhibition of arachidonic acid-dependent nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase activation.[9]

MATERIALS AND METHODS

The study was in two parts. First, we performed a cross-sectional study comparing oxidative stress and NO generation in a population of PAD patients, with healthy

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subjects as the control group. Second, we performed an interventional trial in PAD patients to assess if PLC infusion was able to reduce oxidative stress and in turn enhance NO generation.

Cross-sectional study. The study included 40 consecutive patients, aged 40 to 80 years, who were affected with PAD at Leriche Fontaine stage IIb and had symptoms of intermittent claudication for at least 6 months. Each patient visited as an outpatient in the ambulatory clinic of our division. Forty subjects without clinically relevant atherosclerotic disease were the controls.

PAD was defined according to the following criteria: (1) claudication defined as leg pain on walking, disappearing within 10 minutes on standing, of presumed atherosclerotic origin and (2) an ankle-brachial index (ABI), that was assessed by Doppler ultrasonography as an ankle-arm systolic blood pressure ratio <0.80 on the worst leg at rest.

All patients underwent a full medical history, physical examination, 12-lead electrocardiogram, laboratory tests, measurement of ABI, and a screening treadmill test whereupon the maximal walking distance (MWD) was recorded. Patients had to be in a stable condition, without abrupt changes of walking distance and ABI in the month before the study. They were excluded if they had liver insufficiency, serious renal disorders (serum creatinine >2.8 mg/dL), and myocardial infarction, coronary revascularization, peripheral vascular surgery or percutaneous intervention procedure, unstable angina, acute cerebrovascular disease, or deep venous thrombosis within 6 months before hospital admission. All participants provided written informed consent.

Interventional study with PLC. Ten of the 40 patients were randomly allocated to a 12-hour intravenous infusion of placebo or PLC (6 g/day for 7 days) in a double-blind crossover design. There was a 1-month washout between the two phases of the study. During the study period, all patients were given a similar diet and checked for blood pressure, glycemia, and pulse rate. Also, all patients were nonsmokers and did not train during the entire period of observation. All patients were hospitalized during each period of the study. ABI and treadmill were measured at baseline and 12 hours after the last infusion of placebo or PLC. At baseline and 12 hours after the last infusion, a blood sample was taken to measure serum NO and 8-OHdG. All the analyses were performed blind. The protocol was approved by the regional committee for research ethics. All participants provided written informed consent.

Treadmill test. Treadmill test was performed using a treadmill speed of 3.5 km/h at 12% grade until maximal claudication pain.

ABI measurement. The ABI was calculated with the patient placed in the supine position, measuring the higher systolic pressure of the anterior or posterior tibial artery in each limb, and dividing this pressure with the highest brachial systolic pressure.[10] We performed toe pressure determinations in patients with diabetes. The ABI measurement was obtained 5 minutes before the exercise.

Blood sampling protocol. Subjects underwent routine biochemical evaluation, including fasting, and serum levels of total and high-density-lipoprotein cholesterol, triglycerides, glucose, and insulin. After overnight fasting and supine rest for at least 10 minutes, blood was withdrawn from an antecubital vein.

Serum levels of nitrite/nitrate and 8-hydroxy-2-deoxyguanosine. Blood samples were immediately centrifuged at 3000 rpm for 15 minutes at 4°C, and the supernatant was collected and stored at −80°C until measurement. NO serum levels were evaluated through the measurement of metabolic end products (Calbiochem nitrite nitrate assay, CN Biosciences, San Diego, Calif), nitrite and nitrate (NOx) using enzymatic catalysis coupled with Griess reaction as reported by Verdon.[11] Intra-assay and inter-assay coefficients of variation were 2.2% and 4.0%, respectively.

Serum levels of 8-OHdG were analyzed by using a competitive enzyme-linked immunosorbent assay (Bioxytech 8-OHdG-EIA, OXIS Health Products, Portland, Ore). Intra-assay and inter-assay coefficients of variation were 2.1% and 4.5%, respectively. Day-to-day variation of NO and 8-OHdG was 3% and 3.2%, respectively.

Statistical analysis. Assuming that PLC treatment would reduce serum levels of 8-OHdG by 30%, we postulated that the study sample size should consist of at least 10 patients for each group ($\alpha = 0.05$, $1–\beta = 0.80$). Categoric variables are reported as counts (percentage) and continuous variables as means ± SD, unless otherwise indicated. Independence of categoric variables was tested by χ^2 test. Comparisons between groups were performed by Student's *t* test and were replicated as appropriate with nonparametric tests (Wilcoxon and Kolmogorov-Smirnov *z* test in case of nonhomogeneous variances as verified by Levene's test). Analysis of variance with a Bonferroni test for multiple comparisons was applied in vitro experiments. The correlation analysis was done with Pearson's test. Multiple linear regression analysis was performed by using stepwise selection method. A value of $P < .05$ was considered statistically significant. All analyses were done with SPSS 13.0 software (SPSS Inc, Chicago, Ill).

RESULTS

Cross-sectional study. Clinical characteristics of patients with PAD (Table I) included hypertension in 87.5%, dyslipidemia in 52.5%, diabetes in 32.5%, 60% were ex smokers, and 5% had a clinical history of myocardial infarction (MI). Concomitant treatments included anticoagulants (10%), antiplatelets agents (95%), angiotensin-converting enzyme inhibitors (55%), calcium antagonists (45%), doxazosin (10%), diuretics (55%), oral antidiabetic drugs (25%), insulin (7.5%), and statins (45%).

Compared with controls, patients with PAD had enhanced oxidative stress as documented by elevated serum levels of 8-OHdG (4.4 ± 3.1 ng/mL vs 2.4 ± 1.2 ng/mL; $P < .001$) (Fig1, *A*). NOx generation was significantly reduced in PAD patients compared with controls (11.6 ± 6 μM vs 17 ± 6.1 μM; $P < .001$) (Fig1, *B*). A significant

Table I. Clinical characteristics of patients with PAD and healthy subjects

Variables	*Healthy subjects (n = 40) (%)*	*PAD (n = 40) (%)*	P
Mean age (yr)	64.2 ± 11.2	64.9 ± 7.4	NS
Males/females	30/10	31/9	NS
Hypertension	37.5	87.5	<.001
Diabetes mellitus	0	32.5	<.001
History of MI	0	5	NS
Dyslipidemia	40	52.5	NS
Ex smokers	30	60	.013
Medication			
ACE-inhibitors	25	55	.012
Diuretics	27.5	55	.023
Calcium antagonists	15	45	.007
Doxazosin	5	10	NS
Oral antidiabetic drugs	0	25	.002
Insulin	0	7.5	NS
Antiplatelets agents	0	95	<.001
Anticoagulants	0	10	NS
Statins	7.5	45	<.001
NOx (μmol/L)	17 ± 6.1	11.6 ± 6	<.001
8-OHdG (ng/mL)	2.4 ± 1.2	4.4 ± 3.1	<.001
ABI	1.2 ± 0.11	0.54 ± 0.14	<.001

PAD, Peripheral arterial disease; *MI,* myocardial infarction; *ACE,* angiotensin-converting enzyme; *NS,* not significative; *NOx,* nitrite and nitrate; *8-OHdG,* 8-hydroxy-2-deoxyguanosine; *ABI,* ankle-brachial index.
Data are expressed percentages, a ratio, or as mean ± SD.

inverse correlation was observed between 8-OHdG and NOx ($r = -0.879$; $P < .001$) (Fig 2). To further analyze the relationship between these values, we calculated the NOx/8-OHdG ratio for each patient and found a lower ratio in PAD patients compared with controls (5.9 ± 5.1 vs 16.7 ± 14, $P < .001$).

In patients with PAD, MWD was 79 ± 32 meters. MWD was correlated directly with NOx ($r = 0.36$, $P = .02$) and inversely with 8-OHdG ($r = -0.48$, $P = .002$).

To establish the independent predictors of 8-OHdG in PAD patients, we performed a multivariate linear regression (adjusted for age, ABI, systolic and diastolic blood pressure, smoking history, diabetes and dyslipidemia) showing that NOx (B, −0.410; ES, 0.039; standardized coefficient β, −0.810; $P < .001$) and MWD (B, −0.017; ES, 0.007; standardized coefficient β: −0.194; $P = .017$) were significantly associated. The same statistical analysis (adjusted for age, ABI, systolic and diastolic blood pressure, smoking history, diabetes and dyslipidemia) performed to establish the independent predictors of NOx showed that only 8-OHdG (B, −1.736; ES, 0.152; standardized coefficient β: −0.879; $P < .001$) was significantly associated.

Interventional trial. Clinical characteristics of the patients who participate in the interventional study were similar to the clinical characteristics of the entire PAD population (Table II). In patients given placebo, no changes in MWD (100 ± 29 meters to 103 ± 33 meters; $P > .05$), or in NOx and 8-OHdG serum levels were observed (Fig 3). Conversely in patients given PLC, we observed a significant increase in MWD (101 ± 31 meters to 129 ± 35 meters; $P = .007$) and in serum levels of NOx (14.5 ± 4.5 μM to 17.1 ± 3.8 μM; $P = .007$) (Fig 3). A decrease of 8-OHdG (3.6 ± 1.1 ng/mL to 2.6 ± 1 ng/mL; $P = .005$) was also found (Fig 3). Before ($r = -0.96$; $P < .001$) and after ($r = -0.91$, $P < .001$) PLC treatment, NOx and 8-OHdG serum levels were inversely correlated. Furthermore, we found an increase in the NOx/8-OHdG ratio for each patient from 5.4 ± 3.7 to 9.1 ± 4.6 ($P < .001$).

DISCUSSION

Low generation of NO in patients with PAD may have a deleterious effect in the progression of this vascular pathology because NO is potent inhibitor of platelet function and possesses antiatherosclerotic property that could limit the progression of atherosclerotic plaque. In accordance with previous findings,[5] patients with PAD had low levels of systemic NO that could be dependent upon its reduced synthesis or rapid catabolism. In particular, Boger et al[5] demonstrated that patients with PAD have enhanced levels of asymmetric dimethyl-L-arginine, an endogenous inhibitor of NO synthase, suggesting that in this setting, low NO generation could be due to reduced NO synthesis.

In the present study, we sought to investigate an alternative mechanism that may account for low NO generation. In particular, we analyzed the existence of a relationship between NO generation and oxidative stress that is known to inhibit NO generation. Our findings are in accordance with previous studies[7,8,12] documenting enhanced oxidative stress in patients with PAD. In particular, Bergmark et al[13] demonstrated increased circulating levels of autoantibodies against oxidized low-density lipoprotein in 62 PAD patients with no difference between patients with moderate or severe peripheral atherosclerosis. Mueller et al[7] found elevated serum levels of isoprostanes that were independent predictors of PAD. In 85 PAD patients, Langlois et al[12] detected low serum levels of vitamin C, a known antioxidant, that were independent of smoking habit and hypertension.

The novel finding of the present study is the demonstration of an inverse relationship between oxidative stress and NO, suggesting that in PAD oxidative stress may determine low NO generation. To explore this issue we performed an in vivo study with PLC, a molecule that exerts an antioxidant activity via reduction of arachidonic acid-dependent NADPH oxidase activation.[9]

The study was a double blind placebo-controlled trial in PAD patients to assess if PLC administration was able to interfere with serum levels of 8-OHdG and NO generation. As PLC bioavailability is low by oral administration,[14-16] the trial was performed by intravenous PLC infusion. After PLC administration, we observed a parallel decrease of oxidative stress and increase of NO generation. The fact that an antioxidant treatment was able to enhance NOx serum levels suggests that in PAD patients, oxidative stress is implicated in lowering NO generation.

This hypothesis is consistent with the study by Ruano et al[17] showing that in hypercholesterolemic patients, oxidative stress and NOx were inversely correlated but does

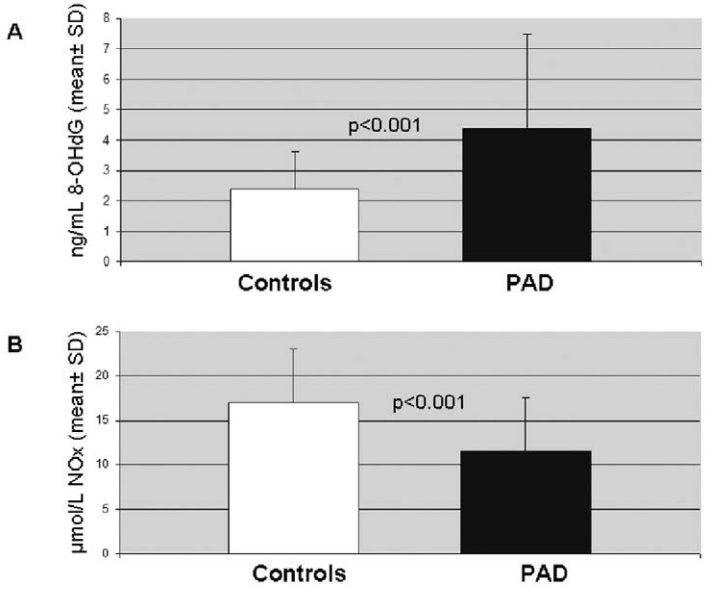

Fig 1. A, Serum levels of 8-hydroxy-2-deoxyguanosine *(8-OHdG)* in patients with peripheral arterial disease *(PAD)* and in control subjects. **B,** Nitrite and nitrate *(NOx)* serum levels in patients with PAD and in control subjects. Data are expressed as mean ± SE.

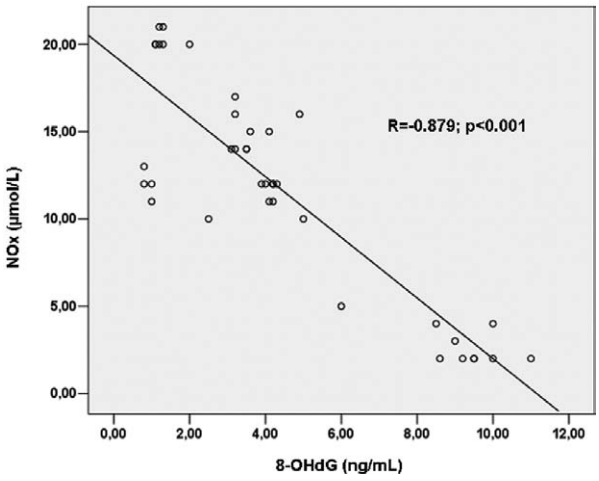

Fig 2. Correlation between nitrite and nitrate *(NOx)* and 8-hydroxy-2-deoxyguanosine *(8-OHdG)* in patients with peripheral arterial disease *(PAD)*.

Table II. Clinical characteristics of patients undergoing interventional trial with propionyl-L-carnitine

N	10
Mean age (year)	62.5 ± 6.6
Males/Females	8/2
Hypertension	100
Diabetes mellitus	30
Dyslipidemia	50
History of MI	0
Ex smokers	70
Medication	
ACE inhibitors	60
Diuretics	40
Calcium antagonists	30
Doxazosin	20
Oral antidiabetic drugs	30
Insulin	0
Anti-platelets agents	100
Anticoagulants	0
Statins	50

MI, Myocardial infarction; *ACE,* angiotensin-converting enzyme.
Data are expressed as percentages, a ratio, or as mean ± SD.

not explain the underlying mechanism. At least two mechanisms may be implicated in the reduction of NOx by PLC. First, the reduction of oxidative stress could result in enhancing NO bioavailability but against this possibility is the fact that NOx may also stem from NO interaction with O_2^-. This hypothesis, however, cannot be fully excluded because NOx can also stem from a mechanism independent of O_2^-.[18,19]

An alternative and more intriguing hypothesis is that oxidative stress reduces NO generation via inhibition of NO synthase[6]; therefore, the reduction of NOx serum levels should be interpreted as a consequence of lowered NO synthesis more than accelerated NO degradation. Further study is therefore necessary to elucidate the intrinsic mechanism through which oxidative stress reduces NO generation in PAD patients.

CONCLUSION

Our findings may have potential clinical implications. Previous studies underscored the relevance of endothelium-derived mediators for vasodilatation in the microcirculation of skeletal muscle. Maxwell et al[20] demonstrated, in partic-

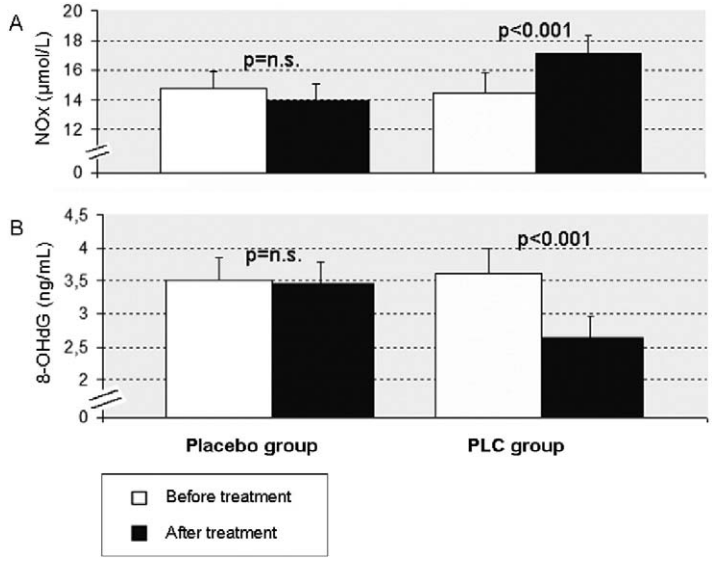

Fig 3. Nitrite and nitrate *(NOx)* and 8-hydroxy-2-deoxyguanosine *(8-OHdG)* serum levels in patients with peripheral arterial disease *(PAD)* before and after intravenous infusion of placebo or propionyl-L-carnitine *(PLC)*. Data are expressed as mean ± SE.

ular, that low NO generation is associated with reduced walking performance through limiting oxygen delivery to skeletal muscle. Also, an interventional trial with L-arginine favored the role of NO in exercise performance.[21] Thus, administration of L-arginine to patients with claudication resulted in a significant increase of MWD.[21,22]

Our data suggest that the use of an antioxidant could represent an alternative approach to cure PAD patients, because the reduction of oxidative stress would result in enhancing NO bioavailability and, eventually, walking performance. This hypothesis could be supported by the inverse correlation between oxidative stress and MWD and by the increase of MWD after PLC treatment. However, the small sample size of interventional trial precludes definite conclusions and suggests that this hypothesis be tested in a larger population.

In conclusion, this study shows that in patients with PAD low NO generation may result from enhanced oxidative stress and suggests that antioxidant treatment is useful to counteract it.

AUTHOR CONTRIBUTIONS

Conception and design: LL, VF
Analysis and interpretation: PP, CR
Data collection: AP, PAA, MV
Writing the article: LL, VF
Critical revision of the article: PP, CR, AP, PAA, MV
Final approval of the article: LL, PP, CR, AP, PAA, MV, VF
Statistical analysis: LL, CR
Obtained funding: VF
Overall responsibility:VF

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